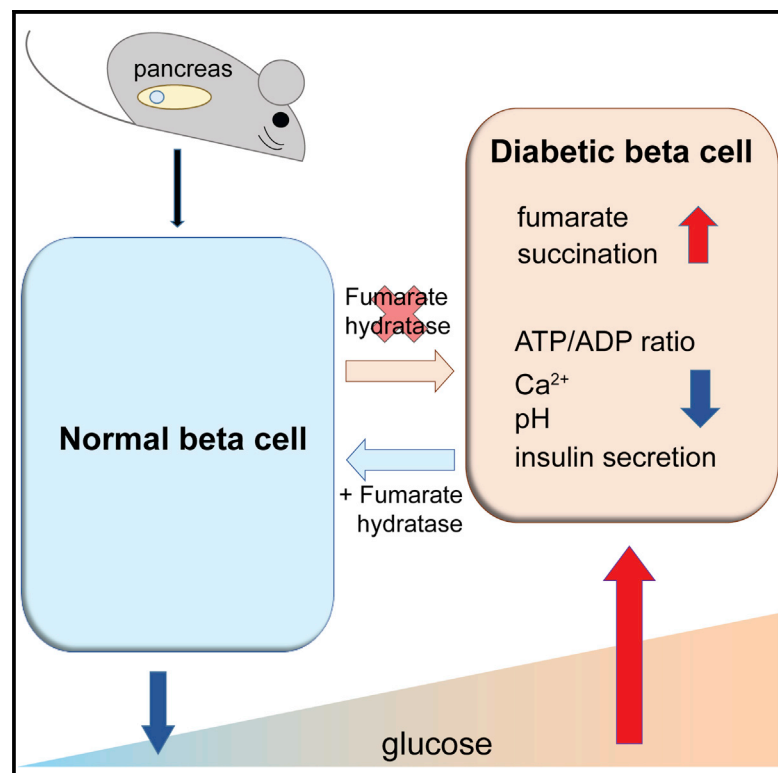


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Fumarate Hydratase Deletion in Pancreatic β Cells Leads to Progressive Diabetes

Graphical Abstract



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In Brief

Adam et al. have shown that progressive diabetes develops if fumarate hydratase is deleted in mouse pancreatic β cells. Such β cells exhibit elevated fumarate and protein succination and show progressively reduced ATP production and insulin secretion. The depleted insulin response to glucose recovers when diabetic islets are cultured in reduced glucose.

Highlights

- Fh1 loss in β cells causes progressive Hif1 α -independent diabetes
- Fh1 loss in β cells impairs ATP generation, electrical activity, and GSIS
- Elevated fumarate is a feature of diabetic murine and human islets
- “Normoglycemia” restores GSIS in Fh1 β KO islets



Fumarate Hydratase Deletion in Pancreatic β Cells Leads to Progressive Diabetes

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SUMMARY

We explored the role of the Krebs cycle enzyme fumarate hydratase (FH) in glucose-stimulated insulin secretion (GSIS). Mice lacking *Fh1* in pancreatic β cells (*Fh1* β KO mice) appear normal for 6–8 weeks but then develop progressive glucose intolerance and diabetes. Glucose tolerance is rescued by expression of mitochondrial or cytosolic FH but not by deletion of *Hif1 α* or *Nrf2*. Progressive hyperglycemia in *Fh1* β KO mice led to dysregulated metabolism in β cells, a decrease in glucose-induced ATP production, electrical activity, cytoplasmic $[Ca^{2+}]_i$ elevation, and GSIS. *Fh1* loss resulted in elevated intracellular fumarate, promoting succination of critical cysteines in GAPDH, GMPR, and PARK 7/DJ-1 and cytoplasmic acidification. Intracellular fumarate levels were increased in islets exposed to high glucose and in islets from human donors with type 2 diabetes (T2D). The impaired GSIS in islets from diabetic *Fh1* β KO mice was ameliorated after culture under normoglycemic conditions. These studies highlight the role of FH and dysregulated mitochondrial metabolism in T2D.

INTRODUCTION

Diabetes is an increasing and serious global health and financial problem (Ashcroft and Rorsman, 2012), characterized by defective insulin secretion from the β cells of the pancreatic islets, which causes elevated blood glucose. Mitochondrial production of ATP plays a key role in glucose-stimulated insulin secretion (GSIS) (Maechler and Wollheim, 1999); an increase in intracellular ATP closes ATP-sensitive K^+ channels (K_{ATP} channels) in the β cell plasma membrane, triggering depolarization and Ca^{2+} -dependent electrical activity. The resulting rise in cytoplasmic Ca^{2+} initiates exocytosis of insulin granules. In addition to this “triggering” effect, glucose amplifies insulin secretion at a stage subsequent to Ca^{2+} influx. Several intracellular factors might mediate this amplifying effect, including ATP, NADPH, and glutamate (Henquin, 2011).

The Krebs-cycle enzyme fumarate hydratase (FH) catalyzes the hydration of fumarate to malate. FH is also a tumor suppressor, mutated in hereditary leiomyomatosis and renal cell cancer (HLRCC) (Launonen et al., 2001). Loss of FH activity results in the intracellular accumulation of fumarate, the stabilization of hypoxia-inducible factor 1 α (HIF1 α), and activation of HIF-dependent pathways, including glucose metabolism (Adam et al., 2014).

The high levels of fumarate that accumulate in FH-deficient cells cause post-translational modification of cysteine residues in proteins to form S-(2-succino)-cysteine (2SC), a process

known as succination (Alderson et al., 2006). This induces loss of activity of the mitochondrial Krebs-cycle enzyme aconitase (Terrette et al., 2013), activation of the antioxidant response sensor nuclear factor (erythroid-derived 2)-like 2 (NRF2) (Adam et al., 2011), and elevation of reactive oxygen species (ROS) signaling (Sullivan et al., 2013; Zheng et al., 2015). Succination has been described in fat and skeletal muscle cells of some diabetic animal models (Nagai et al., 2007; Thomas et al., 2012). Its functional consequences include inactivation of the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Merkley et al., 2014; Blatnik et al., 2008).

HIF1 α is a known regulator of GSIS (Girgis et al., 2012; Spégel et al., 2011). Deletion of von Hippel-Lindau protein (Vhl), an integral component of the HIF1 α degradation pathway, in β cells leads to HIF1 α stabilization, a switch from oxidative to glycolytic metabolism and consequent glucose intolerance (Zehetner et al., 2008; Cantley et al., 2009). The high levels of fumarate that result from FH loss competitively inhibit the 2-oxoglutarate-dependent dioxygenases that catalyze HIF prolyl hydroxylation. This allows HIF to escape degradation and results in the activation of HIF target genes (Isaacs et al., 2005; O'Flaherty et al., 2010), raising the possibility that FH loss might impair insulin secretion via HIF1 α stabilization.

We explored the role of FH in insulin secretion using a mouse model in which *Fh1* was deleted specifically in pancreatic β cells (Fh1 β KO mice). These mice had normal glucose tolerance for the first 6–8 weeks of life, and their β cells had essentially normal properties, including GSIS, despite the lack of a key Krebs-cycle enzyme. However, Fh1 β KO mice subsequently developed rapidly progressing diabetes, culminating in severe glucose intolerance, reduced islet insulin content, and almost complete loss of GSIS.

RESULTS

Mice Lacking *Fh1* in Pancreatic β Cells Exhibit Hif1 α -Independent Glucose Intolerance

We generated animals in which *Fh1* was deleted specifically in pancreatic β cells (Fh1^{fl/fl}Rip2-Cre^{+/-}; Fh1 β KO mice) by intercrossing an *Fh1* conditional knockout mouse (Pollard et al., 2007) with mice expressing Cre recombinase driven by the rat insulin promoter (Rip2-Cre mice; Herrera, 2000). Control (CTL) mice were either Fh1^{fl/fl}Rip2-Cre^{-/-} or Fh1^{fl/+}Rip2-Cre^{+/-} littermates. Deletion of *Fh1* in β cells was confirmed at the protein level in islets of 9- to 12-week-old mice (Figure 1). No marked differences were seen in gross histology between CTL and Fh1 β KO mice in islets (Figures 1A and 1F). Immunohistochemistry (IHC) showed that all cells in CTL islets exhibited uniform expression of FH, which was lost within the core of Fh1 β KO islets. However, some islet cells, most likely α and β cells, retained FH (Figures 1B, 1G, and 1J). *Fh1* loss and elevated fumarate lead to stabilization of HIF1 α and subsequent nuclear localization (Isaacs et al., 2005; Pollard et al., 2005). Figures 1C and 1H show nuclear localization of HIF1 α in most cells of Fh1 β KO islets, but not in CTL islets or the exocrine pancreas. No marked differences were observed in insulin or glucagon IHC between CTL and Fh1 β KO islets (Figures 1D, 1E, 1I, and 1J).

Deletion of *Fh1* was confirmed in Fh1 β KO islets from 9- to 12-week-old mice at the mRNA level. A small amount of *Fh1* mRNA

remained in Fh1 β KO islets, likely reflecting its presence in non- β cells (Figure 1K). Expression of the *Hif1 α* target genes pyruvate dehydrogenase kinase 1 (*Pdk1*) and prolyl hydroxylase dehydrogenase 3 (*Phd3/Egln3*) was increased in Fh1 β KO islets (Figure 1K).

Blood glucose levels were measured in free-fed CTL and Fh1 β KO littermates from 6 to 20 weeks. While young (6–8 weeks of age) Fh1 β KO mice were nearly normoglycemic, they subsequently (at >9 weeks of age) developed severe hyperglycemia (>20 mM) (Figure 1L). Plasma glucose in CTL mice was stable at all ages (<10 mM). For simplicity, here we refer to CTL and Fh1 β KO littermates at 6–8, 9–12, and >15 weeks of age as stages I–III, respectively, to match the progressive diabetes in Fh1 β KO mice. Stages I (non-diabetic [ND]), II (diabetic), and III (severely diabetic) have free-fed blood glucose levels of 12.8 mM \pm 0.6 mM, 22.6 mM \pm 1.2 mM, and 30.4 mM \pm 0.9 mM (Figure 1L). The progression of diabetes was confirmed in glucose tolerance tests, which revealed mild intolerance between 9 and 12 weeks of age (stage II) and severe intolerance by 15 weeks (stage III) (Figures 1M and 1N). No age-dependent deterioration of glucose tolerance was observed in CTL mice.

To assess whether the glucose intolerance of Fh1 β KO mice is mediated by HIF1 α stabilization, we crossed Fh1 β KO and *Hif1 α* ^{fl/m} mice (Cramer et al., 2003) to produce β cell-specific deletion of both *Fh1* and *Hif1 α* (Fh1Hif1 α β KO mice). Deletion of *Hif1 α* , confirmed by loss of HIF1 α staining in all nuclei of Fh1Hif1 α β KO islets (Figure 1O), did not ameliorate the glucose intolerance of Fh1 β KO mice (Figure 1P). Loss of *Fh1* also leads to stabilization of NRF2 and activation of downstream pathways, typified by increased expression of *Hmox1* (confirmed in Figure S1D) (Adam et al., 2011). The role of *Nrf2* in β cell function is unclear, proposed to protect from oxidative damage and blunt GSIS (Urano et al., 2013). To determine the contribution of *Nrf2* to glucose homeostasis, we crossed Fh1 β KO with a constitutive knockout of *Nrf2* (Itoh et al., 1997) to delete both *Fh1* and *Nrf2* in the β cell (Fh1 β Nrf2DKO). The glucose intolerance of Fh1 β KO mice was unaltered in the Fh1 β Nrf2DKO mice (Figure S1A). In contrast, crossing Fh1 β KO mice with mice stably expressing either full-length human FH (Fh1 β KO+FH) or cytoplasmic FH (FH^{cyt}) (Fh1 β KO+FH^{cyt}) (Adam et al., 2013) fully reversed the glucose intolerance tested in stage III mice and restored the mRNA expression of *Egln3* and *Hmox1* (Figures 1Q and S1B–S1D), and these mice were normoglycemic for >1 year.

Deletion of *Fh1* in β Cells Results in Progressive Loss of GSIS

To explore the cause of glucose intolerance in Fh1 β KO mice, we examined insulin secretion from the perfused pancreas. Glucose elevation from 1 mM to 6 mM transiently stimulated insulin release \sim 10-fold in stage II CTL mice but not in Fh1 β KO littermates (Figure 2A). Nevertheless, the response to 20 mM glucose was almost superimposable in both genotypes. In stage III Fh1 β KO mice, the response to both 6 mM and 20 mM glucose was 85% less than in CTL (Figure 2B). Similar effects on GSIS were obtained in static incubations (Figure S2). The mitochondrial substrate α -ketoisocaproic acid (α -KIC) stimulated insulin secretion \sim 5-fold in CTL islets but had no effect in Fh1 β KO islets (Figure S2).

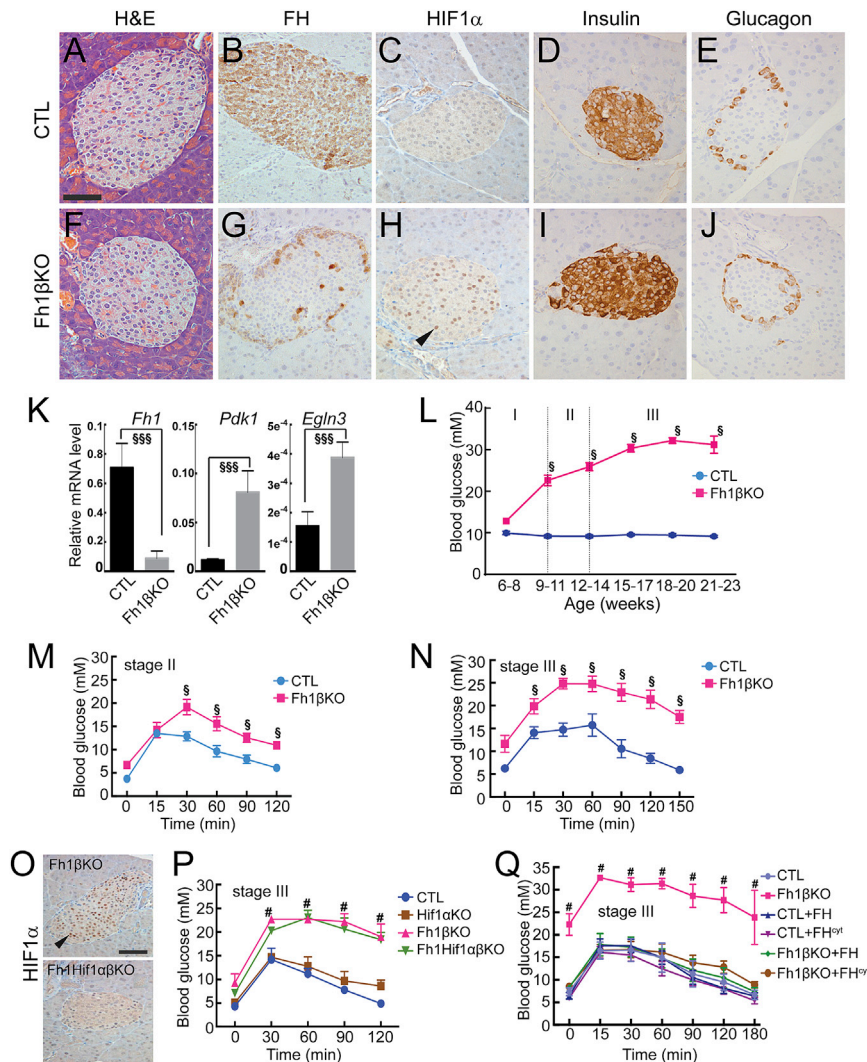


Figure 1. *Fh1* Loss in Pancreatic β Cells Results in Progressive *Hif1* α -Independent Glucose Intolerance

(A–J) Histological analysis of pancreatic islets from stage II CTL (A–E) and Fh1 β KO (F–J) mice (n = at least 10 islets from each of 10 mice per genotype). (A and F) H&E staining of CTL and Fh1 β KO, respectively. (B–E and G–J) Immunohistochemistry (IHC) for FH (B and G), HIF1 α (C and H), insulin (D and I), and glucagon (E and J) in CTL (B–E) and Fh1 β KO (G–J). Scale bar, 50 μ m (all panels). (K) mRNA levels of *Fh1*, *Pdk1*, and *Egln3* relative to *Actb* (β -actin) in stage II CTL (black) and Fh1 β KO islets (gray). (n = 30–50 islets in 5 experiments from a total of 10 animals of each genotype). *** p < 0.0001.

(L) Free-fed blood glucose measured in different aged CTL (blue) and Fh1 β KO mice (red). Stages I, II, and III are identified (n > 200 Fh1 β KO mice, and n > 50 CTL mice). § p < 0.0001.

(M and N) Intraperitoneal glucose tolerance test (IPGTT) performed in (M) stage II CTL (blue; n = 15) and Fh1 β KO (red; n = 10) mice and (N) stage III mice (n = 7 per group). § p < 0.05.

(O) IHC for HIF1 α in pancreas of stage III Fh1 β KO (top) and Fh1Hif1 α βKO (bottom) mice (n = at least 10 islets from each of 4 mice per genotype). Scale bar, 50 μ m.

(P) IPGTT performed in stage III CTL, Fh1 β KO, Hif1 α KO, and Fh1Hif1 α βKO mice. # p < 0.05, comparing Fh1 β KO or Fh1Hif1 α βKO versus CTL or Hif1 α KO; Fh1 β KO versus Fh1Hif1 α βKO and CTL versus Hif1 α KO are not significant (n = at least 5 mice per genotype).

(Q) Reintroduction of FH or FH^{cyt} rescued the glucose intolerance of Fh1 β KO mice. IPGTT performed on stage III CTL, Fh1 β KO, CTL+FH, CTL+FH^{cyt}, Fh1 β KO+FH, and Fh1 β KO+FH^{cyt} mice. # p < 0.0001, Fh1 β KO versus all other groups (n = 6–8 mice per genotype).

Arrows in (H) and (O) point to nuclei. Error bars represent \pm SEM. See also Figure S1.

These differences in insulin release correlated with a reduction in insulin content. In islets from stage II mice, insulin content and insulin granule density were reduced by \sim 50% in Fh1 β KO islets (Figures 2C–2E). Pancreatic insulin content was 97% less in stage III Fh1 β KO mice, compared to CTL littermates (Figure 2F), and there was a marked decrease in insulin-positive cells (Figure 2G). When insulin secretion data are normalized to basal values, secretion from stage II Fh1 β KO islets remains strongly reduced, suggesting that there is a functional defect (Figure S2).

Fh1 β KO Islets Exhibit Dysregulated Metabolism

As mitochondrial metabolism plays a key role in GSIS (Ashcroft and Rorsman, 2012) and *Fh1* deletion disrupts the Krebs cycle, we analyzed glucose utilization (3 H₂O production, reflecting combined flux through the glycolytic and pentose phosphate pathways) and oxidation (CO₂ production, reflecting mitochondrial metabolism) (Hellman et al., 1971). In both CTL and Fh1 β KO stage II islets, glucose oxidation increased 4-fold when glucose was increased from 1 to 20 mM (Figure 3A). Glucose utilization

also increased 4-fold, but the effect was 35%–40% greater in Fh1 β KO than CTL islets (Figure 3B), suggestive of increased aerobic glycolysis (O’Flaherty et al., 2010).

Metabolite analysis in islets from stage II mice confirmed an overall pattern of changes similar to that observed in other FH-deficient cells (Adam et al., 2011; Frezza et al., 2011). In particular, levels of fumarate, argininosuccinate, and adenylosuccinate were increased, and aspartate was reduced (Figure 3C; Table S1), indicating that FH loss leads to stimulation of the urea cycle/arginine biosynthesis pathway (Adam et al., 2013; Zheng et al., 2013). Levels of AMP, uridine monophosphate (UMP), and guanosine monophosphate (GMP) were decreased, suggesting both purine and pyrimidine metabolism are compromised as a consequence of aspartate depletion, but likely reflect altered adenylosuccinate metabolism due to elevated fumarate (Bulusu et al., 2011). Importantly, no difference was observed in oxidized or reduced glutathione between CTL and Fh1 β KO islets, suggesting no major change in ROS or ROS signaling (Sullivan et al., 2013). Islet fumarate and argininosuccinate content

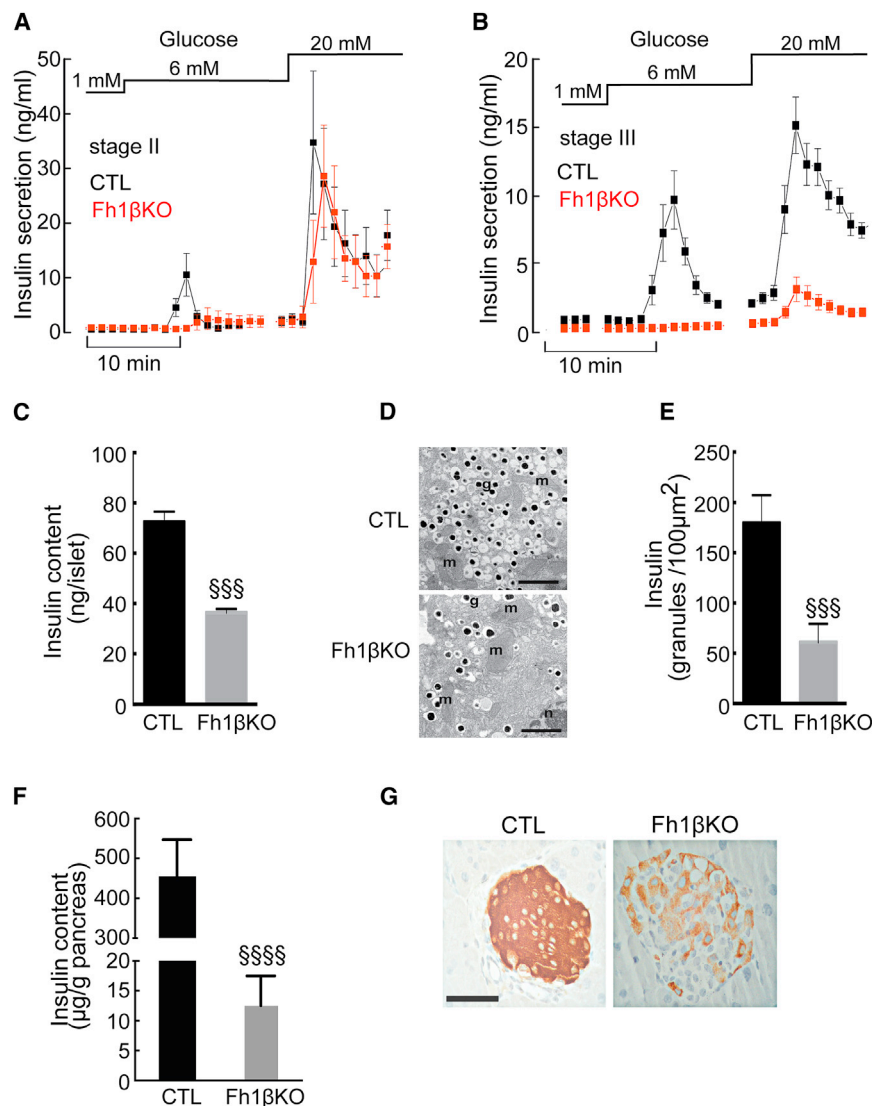


Figure 2. Deletion of *Fh1* in β Cells Impairs Insulin Secretion

(A and B) Insulin secretion from the perfused pancreata of stage II (A) and stage III (B) Fh1 β KO mice (red) and CTL mice (black) (n = 3 mice for each genotype and age) in response to 1, 6, and 20 mM glucose. Statistical significances are omitted for clarity.

(C) Insulin content of islets from stage II CTL mice (black; n = 10 experiments; n = 10 mice) and Fh1 β KO mice (gray; n = 14 experiments; n = 22 mice). ****p < 0.0001.

(D) Electron micrographs of β cells from CTL and Fh1 β KO mice. Abbreviations: g, insulin secretory granules; m, mitochondrion; n, nucleus. Scale bars, 500 nm.

(E) Insulin granule density measured in electron micrographs of β cells from stage II Fh1 β KO (gray) and CTL (black) mice (25–30 β cells in 3–5 islets per genotype). ***p < 0.001.

(F) Pancreatic insulin content of stage III CTL (black, n = 5) and Fh1 β KO (gray, n = 10) mice. ****p < 0.0001.

(G) Representative examples of insulin IHC in stage III Fh1 β KO and CTL islets (n > 100 islets from n > 10 mice of each genotype). Scale bar, 50 μ m. Error bars represent \pm SEM. See also Figure S2.

were normalized following re-expression of either FH or FH^{cyt} in mice (Figures S3A and S3B).

The lack of FH means there is a loss of Krebs-cycle intermediates for every glucose molecule entering the mitochondria. Since metabolism might be maintained by enhanced utilization of amino acids (anaplerosis), we measured 16 key amino acids in islets at both 1 mM and 20 mM glucose. Glucose elevation increased levels of 15 amino acids in CTL islets but had little effect in stage II Fh1 β KO islets (Figures 3D, S3C, and S3D).

Glutamate may play a key role in both glucose- and incretin-induced insulin secretion, via the amplifying (non-K_{ATP}-dependent) pathway (Maechler and Wollheim, 1999). Because the glucose-induced increase in glutamate was lower in Fh1 β KO islets than in CTL islets (Figure 3D), we investigated the amplifying pathway of insulin secretion using islets from stage II animals depolarized with 70 mM extracellular K⁺ (to test the amplifying effect of glucose). Increasing glucose from 2 mM to 20 mM amplified insulin secretion by 600% in CTL islets but only by

that the acute increase of intracellular fumarate in Fh1 β KO islets is not the cause of the impaired GSIS.

Some aspects of altered metabolism in Fh1 β KO islets were also investigated by comparing glutamine (m0 to m+5) and glucose (m0 to m+6) isotopomers in islets from stage II Fh1 β KO and CTL mice incubated in 1 mM or 20 mM glucose with either [U-¹³C₅]-glutamine or ¹³C₆-glucose (Figure S4). Increased levels of fumarate and argininosuccinate, derived from glutamine, were observed in islets subsequently exposed to 20 mM glucose (Figures S4C and S4D). Islets cultured at 20 mM glucose utilized glutamine to generate uridine 5'-diphosphate (UDP)-N-acetylglucosamine (Figures S4H and S4M), a precursor for synthesis of glycosaminoglycans, proteoglycans, and glycolipids (Bond and Hanover, 2013). Substitution of the isotopomers m+2, m+3, m+4, and m+5 for the naturally occurring m+0 and m+1 forms was increased significantly in both glutamate and aspartate in both CTL and Fh1 β KO islets incubated with 20 mM glucose (Figures S4J, S4L, S4N, and S4O).

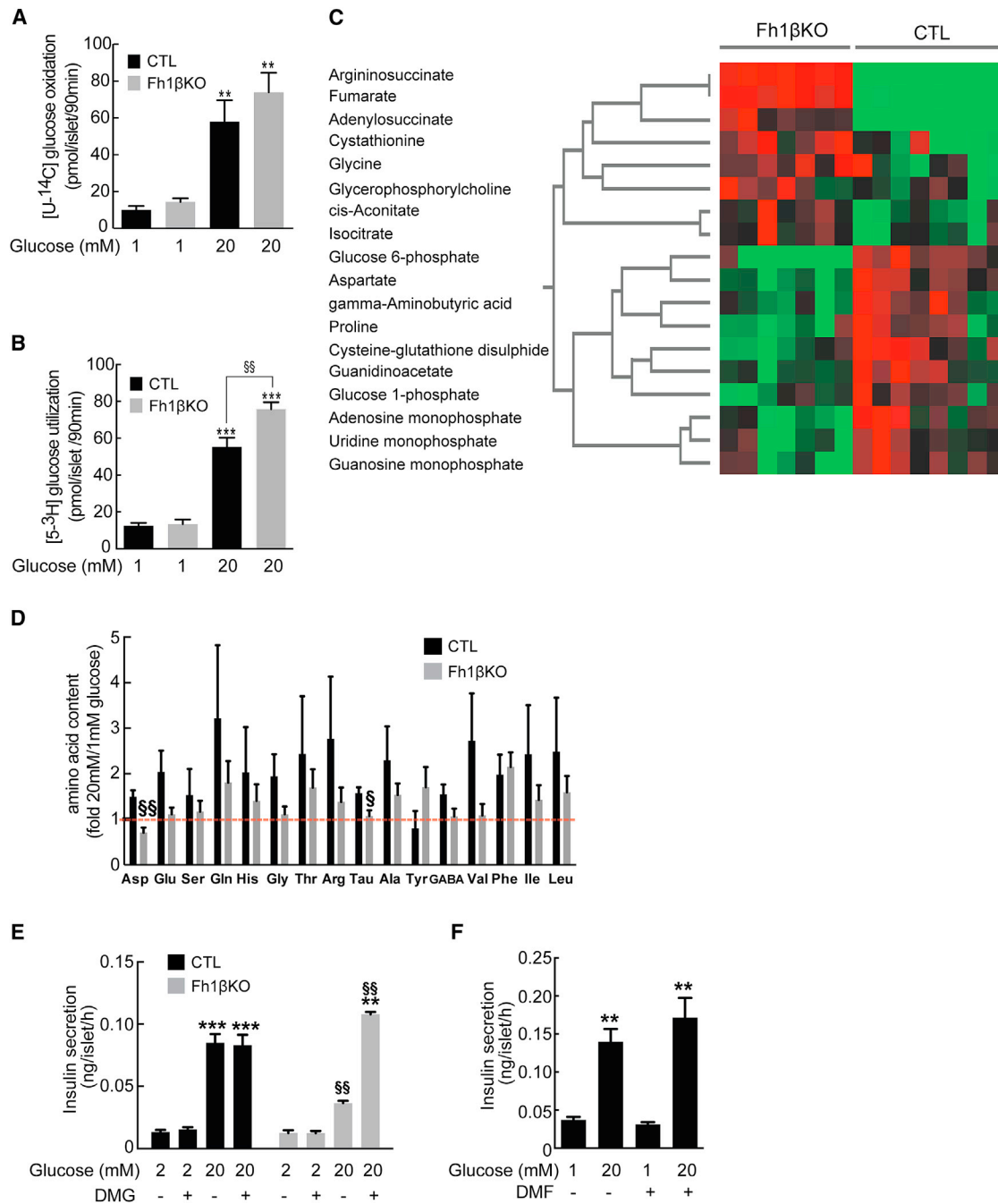


Figure 3. Ablation of *Fh1* Causes Dysregulated Metabolism

(A) Glucose oxidation in stage II Fh1 β KO and CTL islets ($n = 7$ CTL and $n = 16$ Fh1 β KO mice in 3 experiments) at 1 mM and 20 mM glucose. ** $p < 0.01$ versus 1 mM glucose; not significant between CTL and Fh1 β KO islets.

(B) Glucose utilization in CTL and Fh1 β KO islets from stage II mice ($n = 7$ mice of each genotype in 3 experiments) at 1 mM and 20 mM glucose. *** $p < 0.001$ versus 1 mM glucose; §§ $p < 0.01$ between CTL and Fh1 β KO at 20 mM glucose.

(C) Heatmap of metabolites (measured by CE-TOFMS) that show significant concentration differences between islets from stage II CTL ($n = 8$ mice) and Fh1 β KO ($n = 7$ mice) mice incubated at 5 mM glucose for 1 hr. Red and green for Fh1 β KO islets indicate metabolites that are significantly increased or decreased, respectively, versus CTL islets ($p < 0.05$); Student's t test. Each column represents values for islets from one animal. Absolute values are given in Table S1. Branch points indicate metabolites linked in common pathways.

(D) Amino acid content in islets of stage II Fh1 β KO (gray; $n = 12$) and CTL (black; $n = 8$) mice at 20 mM glucose, expressed relative to that at 1 mM glucose (20G/1G) ($n = 30$ islets per group analyzed in triplicate in 5 experiments). Abbreviations: Asp, aspartate; Glu, glutamic acid; Ser, serine; Gln, glutamine; His, histidine; Gly,

(legend continued on next page)

Progressive Loss of GSIS in Fh1 β KO Mice Correlates with Reduced ATP Production and β Cell Electrical Activity

Next, we compared the effects of glucose on ATP production and electrical activity in CTL and Fh1 β KO mice. The glucose-induced increase in the ATP/ADP ratio was identical in stage I Fh1 β KO and CTL islets (Figure 4A). However, β cells in islets from stage III Fh1 β KO mice exhibited far smaller glucose-induced increases in the ATP/ADP ratio than age-matched CTL β cells (Figures 4B and 4C).

Glucose-induced electrical activity in β cells from stage II Fh1 β KO mice was similar to that of CTL islets (Figure 4D). However, clear differences were observed in stage III mice. Whereas CTL β cells responded to 20 mM glucose with membrane depolarization and action potential firing, β cells in stage III Fh1 β KO islets were refractory to glucose stimulation (Figure 4E). In such glucose-unresponsive Fh1 β KO β cells, electrical activity was elicited by the K_{ATP} -channel blocker tolbutamide. Together, these data suggest that glucose is unable to inhibit K_{ATP} channels in stage III Fh1-deficient β cells, because ATP generation is reduced.

Fh1 Deletion Causes Disruption of β Cell Mitochondrial Ultrastructure

The morphology, size, and distribution of mitochondria in β cells of stage II mice were analyzed by electron microscopy. Whereas mitochondria in CTL β cells had normal morphology with clear cristae, many mitochondria in Fh1 β KO β cells were swollen without clear cristae, and some were very large ($>1.25\ \mu\text{m}$ in diameter). There was larger range in the mitochondrial area in Fh1 β KO β cells, suggesting an imbalance between mitochondrial fission and fusion (Figures S5A and S5B). Mitochondria in islets from Fh1 β KO+FH (full-length FH rescue) or Fh1 β KO+FH^{cyt} (cytoplasmic FH rescue) mice were similar to those of CTL mice (Figure S5B).

Protein Succination in Diabetic Islets

Fumarate reacts with cysteine residues in proteins in a non-enzymatic process known as succination. Using an antibody (2SC) that labels succinated proteins specifically (Blatnik et al., 2008), we confirmed that succination was detected in Fh1 β KO β cells and not in CTL β cells (Figures 5A and 5B).

Culture of adipocytes in high glucose elevates fumarate and results in protein succination (Nagai et al., 2007). Therefore, we determined quantitatively whether hyperglycemia would increase fumarate in islets. Fumarate levels were ~ 100 -fold higher in stage II Fh1 β KO islets, even after 1 hr incubation at 1 mM glucose, yet they were further increased at 20 mM glucose (Figure 5C). Glucose also increased fumarate levels in CTL islets; 1 hr

incubation at 20 mM glucose increased fumarate levels 12-fold, compared to islets incubated at 1 mM glucose. This was not due to a reduction in FH (Figure 5D) and, thus, is likely a consequence of the enhanced glucose metabolism. Similar acute (1 hr) effects of high glucose on fumarate content were observed in ND human islets (Figures 5E and 5F). Interestingly, the fumarate content of islets from donors with type 2 diabetes (T2D) was higher than that in ND islets, and high glucose produced no further increase (Figure 5F).

Tandem mass spectrometry (MS/MS) analysis of islets from stage II Fh1 β KO mice islets detected succination of key cysteine residues in glyceraldehyde 3-phosphate dehydrogenase (GAPDH; residue C150) (Figures 5G and 5H), guanosine monophosphate reductase (GMPR; C186) (Figure S6A; Table S2), and Parkinson's disease (autosomal recessive, early onset) 7 (PARK 7/DJ-1; C106). Succination of PARK7/DJ-1 (C106) was also observed in islets from a human T2D donor (Figure S6B; Table S2).

Succination of residue C150 of GAPDH was increased by 270% in Fh1 β KO islets (Figures 5G and 5H). Succination of GAPDH, detected in the gastrocnemius muscle of diabetic rats, has been shown to reduce enzyme activity (Blatnik et al., 2008). This might limit glucose flux through glycolysis, leading to accumulation of upstream glycolytic 3- and 6-carbon intermediates. We tested the possible functional consequences by culturing CTL islets for 24 hr in the presence of 10 mM of the triose D-glyceraldehyde. This resulted in the complete loss of GSIS and correlated with a slight increase in islet insulin content (Figures S7A and S7B).

Impact of Fh1 Deletion on Cytosolic Calcium and pH

Links have been proposed between glucose and pH_i (Shepherd and Henquin, 1995). The loss of glucose- and Ca^{2+} -dependent electrical activity in stage III Fh1 β KO β cells is predicted to cause an alteration in Ca^{2+} handling. Intracellular accumulation of fumarate, the anion of fumaric acid, may lower cytoplasmic pH (pH_i) and thereby compromise GSIS, effects compounded by the inhibition of GAPDH and accumulation of acidic trioses. Therefore, we performed parallel measurements of $[\text{Ca}^{2+}]_i$ and pH_i in stages I and III Fh1 β KO β cells and CTL cells. Changes in $[\text{Ca}^{2+}]_i$ were similar in stage I CTL and Fh1 β KO β cells (Figures 6A and 6B). Thus, glucose, glyceraldehyde, and high K^+ -induced membrane depolarization increased $[\text{Ca}^{2+}]_i$. Tolbutamide had little additional stimulatory effect on $[\text{Ca}^{2+}]_i$ when tested in the presence of 20 mM glucose (cf. Figure 4E). Although basal pH_i was slightly lower in β cells from stage I Fh1 β KO mice than in CTL cells (as expected from the elevated fumarate), the responses to glucose and glyceraldehyde were similar (Figures 6A, 6B, and 6E): glucose and glyceraldehyde lowered pH_i

glycine; Thr, threonine; Arg, arginine; Tau, taurine; Ala, alanine; Tyr, tyrosine; GABA, gamma aminobutyric acid; Val, valine; Phe, phenylalanine; Ile, isoleucine; Leu, leucine.

(E) Insulin secretion from islets from stage II Fh1 β KO (gray) and CTL (black) islets ($n = 4$ experiments; $n = 3$ mice per genotype) in the presence of 70 mM KCl and 2 mM or 20 mM glucose and 5 mM dimethylglutamate (DMG), as indicated. $^{**}p < 0.01$ versus 2 mM glucose in Fh1 β KO; $^{***}p < 0.0001$ versus 2 mM glucose (CTL); $^{SS}p < 0.01$ CTL versus Fh1 β KO in 20 mM glucose.

(F) Insulin secretion in wild-type islets ($n = 13$ experiments; $n = 3$ mice) at 1 mM and 20 mM glucose with the addition of dimethyl fumarate (5 mM DMF). $^{**}p < 0.001$ or better versus 1 mM glucose.

Error bars represent \pm SEM. See also Figures S3 and S4 and Table S1.

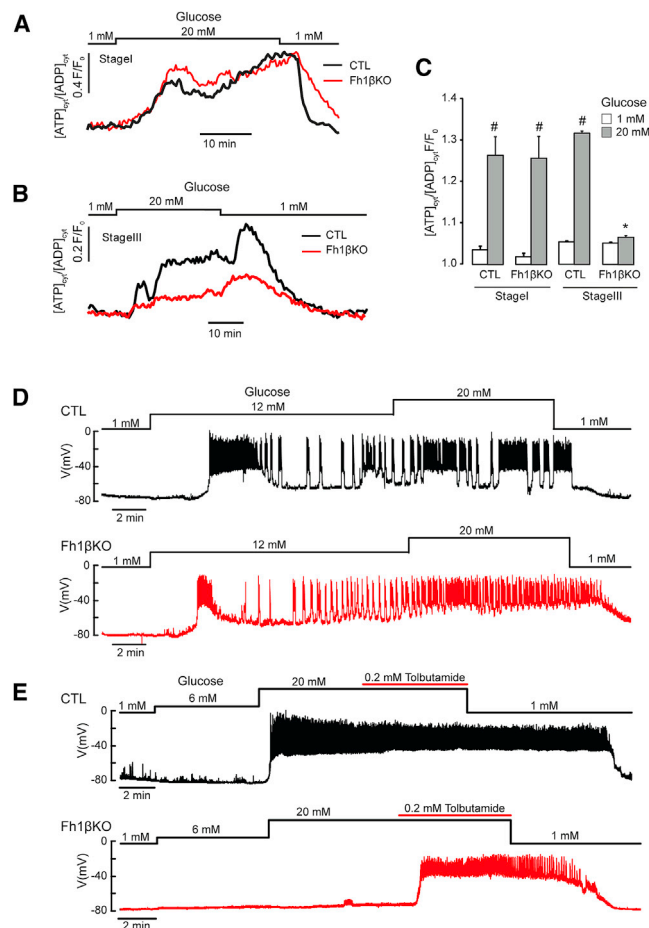


Figure 4. Fh1 β KO β Cells Exhibit Impaired Electrical Activity and Reduced ATP Production with Age

(A) Glucose-induced changes in the ATP/ADP ratio in β cells from stage I CTL (black) and Fh1 β KO (red) mice. Each trace is the average of >200 cells. (B) Same as in (A) but using islets from stage III CTL and hyperglycemic Fh1 β KO mice. Each trace is the average of >200 cells. (C) Cytoplasmic ATP/ADP ratio at 1 mM and 20 mM glucose in islets from stage I and stage III Fh1 β KO and age-matched CTL mice ($n = 4$ mice per genotype, >200 cells per mouse). Responses are normalized to the ratio at 1 mM glucose. $^*p < 0.05$ versus CTL; $^{\#}p < 0.05$ versus basal (3 mM glucose). Error bars represent \pm SEM. (D) Glucose-induced electrical activity in β cells of stage II CTL or Fh1 β KO β cells. Traces are representative of 5 (CTL) or 4 (Fh1 β KO) β cells from at least 3 mice of each genotype. (E) Same as in (D) but using islets from stage III CTL and Fh1 β KO mice. Traces are representative of 5 (CTL) or 4 (Fh1 β KO) β cells from at least 3 mice of each genotype.

reversibly in both CTL and Fh1 β KO β cells, and high-[K $^+$] $_o$ depolarization produced a further drop in pH $_i$.

The [Ca $^{2+}$] $_i$ and pH $_i$ responses of CTL β cells did not change with age, in contrast to the responses of those of stage III Fh1 β KO mice (Figures 6C–6E). Both glucose and D-glyceraldehyde were without effect on [Ca $^{2+}$] $_i$, and tolbutamide was only effective in some β cells. However, high [K $^+$] $_o$ depolarization (which bypasses metabolism) consistently increased [Ca $^{2+}$] $_i$. Basal pH $_i$ was lower in stage III Fh1 β KO β cells than either CTL

or stage I Fh1 β KO β cells (Figures 6C–6E). Increasing glucose to 20 mM or the addition of 10 mM D-glyceraldehyde reduced pH $_i$ in CTL β cells but did not cause further acidification in stage III Fh1 β KO β cells (Figures 6D and 6E).

Impaired GSIS in Fh1 β KO Islets Is a Consequence of Hyperglycemia

We reasoned that the deterioration of GSIS in Fh1 β KO islets over time might be a consequence of the progressive hyperglycemia (Brereton et al., 2014). We tested whether the impairment of GSIS could be reversed. Freshly isolated islets from stage III Fh1 β KO mice were refractory to 6 mM glucose, and the response to 20 mM glucose was only 30% of that seen in CTL islets (Figure 7A), echoing the data from the perfusion experiments (Figure 2B).

After culture at 12 mM glucose for ~65 hr, secretory responses in CTL islets were essentially the same as in freshly isolated islets. However, GSIS at 20 mM glucose was dramatically improved in Fh1 β KO islets and approached that of the CTL islets (Figure 7B). Insulin content was 35% lower in Fh1 β KO than in CTL islets (Figure 7C).

Finally, we tested whether the loss of GSIS in stage III Fh1 β KO islets reflects a direct “glucotoxic” effect or is related to accelerated glucose metabolism. Distinct from what was seen following culture at 12 mM glucose (Figures 7A and 7B), islets from stage III Fh1 β KO mice cultured for 3 days at 20 mM glucose showed no recovery of GSIS. By contrast, islets cultured in the presence of the glucokinase inhibitor mannoheptulose (Zelent et al., 2005; Coore and Randle, 1964) exhibited some (limited) glucose responsiveness (Figure 7D).

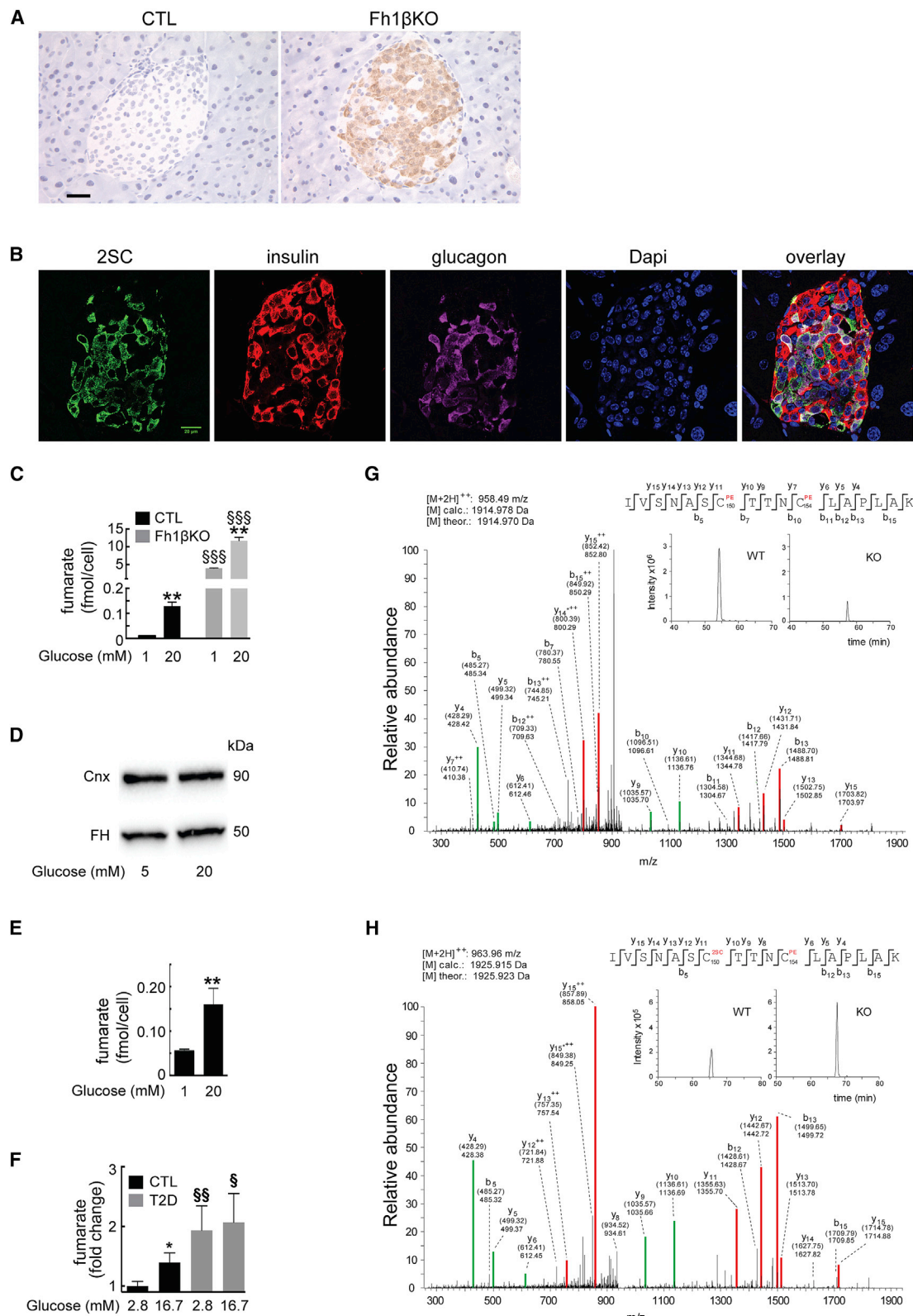
DISCUSSION

We show here that lack of the Krebs-cycle enzyme FH causes a progressive deterioration of β cell function, resulting in severe diabetes associated with impaired oxidative metabolism, ATP production, intracellular calcium handling, and cytosolic acidification.

Distinct from the mouse model in which *Vhl* is deleted in β cells (Zehetner et al., 2008; Cantley et al., 2009), we found that the diabetes associated with *Fh1* loss is *Hif1 α* and *Nrf2* independent. Our data are consistent with earlier observations that, while *Fh1* deletion leads to the accumulation of fumarate, and HIF1 α is stabilized, many associated functional changes are independent of HIF1 α (O’Flaherty et al., 2010; Adam et al., 2013; Ternette et al., 2013). Similar to previous studies, we were unable to detect HIF1 α in normal β cells, and deletion of *Hif1 α* alone did not disrupt glucose homeostasis (Zehetner et al., 2008; Cantley et al., 2009). However, it remains unclear whether β cell dysfunction and diabetes are caused by HIF1 α stabilization or vice versa (Cantley et al., 2010; Girgis et al., 2012). In the Fh1 β KO mouse, loss of glucose regulation appears entirely attributable to FH. The hyperglycemia that develops may drive HIF1 α stabilization, perhaps compounding GSIS impairment further in a feedback loop.

Why Do Young (Stage 1) Fh1 β KO Mice Have Normal Glucose Tolerance?

Given the importance of mitochondrial metabolism for insulin secretion, the mild phenotype of stage I Fh1 β KO mice was



(legend on next page)

surprising, not least as *Fh1* deletion led to >100-fold accumulation of fumarate, consistent with arrest of the Krebs cycle at FH. Because insulin secretion was unimpaired, this suggests that re-filling of Krebs-cycle intermediates (anaplerosis) must occur distal to FH. Labeling experiments in mouse embryonic fibroblasts (MEFs) lacking *Fh1* show that fumarate exits the mitochondria to the cytoplasm but is then metabolized to aspartate via the urea cycle, which re-enters the Krebs cycle (Adam et al., 2013). Studying metabolism in murine pancreatic islets is difficult because of the limited amounts of material available, especially from the diabetic islets. Our analyses of Fh1 β KO islets by capillary electrophoresis time of flight MS (CE-TOFMS) indicate similar disruption of the urea cycle and purine metabolism and explain, in part, why the diabetic phenotype of Fh1 β KO mice can be rescued by re-expression of cytosolic FH. Moreover, Fh1 β KO islets exhibit increased utilization and oxidation of glucose, consistent with aerobic glycolysis, recapitulating previous observations made when *Fh1* was deleted in MEFs and mouse kidney (O'Flaherty et al., 2010; Adam et al., 2013).

Normally, excess Krebs-cycle reactants are used to produce amino acids via cataplerosis (Choi et al., 2011; MacDonald et al., 2005). However, in Fh1 β KO islets, amino acid content tended to be reduced, and glucose did not increase the content of several amino acids; for example, glutamate. This is of interest because glutamate has been implicated in the amplification of GSIS (Maechler and Wollheim, 2000). Exogenous dimethyl glutamate restored GSIS in Fh1 β KO islets but had no effect in CTL islets. These data support the idea that glutamate functions as an "amplification signal." Another possibility is that exogenous glutamate may restore GSIS by feeding into the Krebs cycle and partially restoring mitochondrial metabolism (Frezza et al., 2011).

Despite the ~50% reduction in insulin content, the response to 20 mM glucose was maintained in stage II Fh1 β KO mice. We speculate that Fh1 β KO β cells may compensate for the reduction of insulin content by stimulation of insulin exocytosis. Fh1 β KO islets contain significantly elevated levels of adenylosuccinate, which has been proposed to stimulate exocytosis

by inhibition of the sentrin/SUMO-specific protease 1 (Gooding et al., 2015).

Rapid Progression to Diabetes in Stage II Fh1 β KO Mice

Once slight hyperglycemia developed, rapid deterioration of glycemic control was observed (up to 10 mM/week). We speculate that a small elevation of plasma glucose induces a vicious cycle of impaired insulin secretion and hyperglycemia. In part, this arises because, as blood glucose increases, more glucose enters metabolism, causing even greater stress on the β cell with increased fumarate levels. It is possible that metabolism of cytosolic fumarate and anaplerosis eventually become unable to maintain the Krebs cycle and that, once this occurs, rapid deterioration of both GSIS and glucose tolerance occurs.

Why Do Fh1 β KO Mice Become Glucose Intolerant?

Our results indicate that Fh1 β KO mice eventually develop diabetes because glucose is no longer able to stimulate insulin secretion. This is because of a failure of mitochondrial metabolism that culminates in impaired ATP production, defective K_{ATP} -channel closure, and suppression of electrical activity and $[Ca^{2+}]_i$.

Why does ATP production fail? Pumping H^+ across the inner mitochondrial membrane, the electron transport chain produces the alkalization of the mitochondrial matrix (Wiederkehr et al., 2009) and membrane hyperpolarization, which are needed to drive ATP synthesis in β cells. Fumaric acid ($C_4H_4O_4$) is an acid with two hydroxyl groups with pKa of 3.0 and 4.4; thus, loss of FH activity can accordingly be expected to result in acidification not just of the cytoplasm (experimentally measured) but also of the mitochondrial matrix.

There is a sigmoidal relationship between glucose concentration and mitochondrial metabolism (Ashcroft et al., 1970; Hellman et al., 1971). At low glucose concentrations, the Krebs cycle runs at a fairly low rate, and the amount of fumarate generated and deposited in the cytosol will be low. Thus, if Fh1 β KO mice remain normoglycemic, cytoplasmic acidification will be modest. In agreement with this idea, glucose-responsive β -cells

Figure 5. Succination Is a Feature of Elevated Fumarate

(A) IHC for 2SC in islets from stage II CTL (left) and Fh1 β KO (right) islets ($n > 300$ islets from 10 mice of each genotype). Scale bar, 20 μ m.

(B) Immunofluorescence for 2SC (green), insulin (red), glucagon (purple), nuclei (blue), and overlay in a representative islet from a stage III Fh1 β KO mouse ($n > 100$ islets from $n > 10$ Fh1 β KO mice). Scale bar, 20 μ m.

(C) Fumarate content determined by CE-TOFMS in islets from stage II Fh1 β KO mice (gray; all islets from $n = 6$ mice) and CTL mice (black; all islets from $n = 6$ mice) incubated for 1 hr at 1 mM or 20 mM glucose. ** $p < 0.01$ versus 1 mM glucose; \$\$\$ $p < 0.01$ versus CTL.

(D) Western blot of FH protein in islets from stage II CTL mice (all islets from $n = 3$ mice) cultured for 24 hr at 5 mM or 20 mM glucose. Cnx, calnexin, loading control.

(E) Fumarate content measured by CE-TOFMS of islets from human ND donors (4 donors) incubated for 1 hr at 1 mM or 20 mM glucose. ** $p < 0.01$ versus 1 mM glucose.

(F) Fumarate content determined by gas chromatography-mass spectrometry (GC-MS) in human ND (31 donors) or T2D (7 donors) islets cultured at 2.8 mM and 16.7 mM glucose. Content is expressed relative to that of ND islets at 2.8 mM glucose.

(G and H) MS/MS spectra for the GAPDH-derived peptide IVSNASCTTNCLAPLAK in its non-succinated (G) and succinated (H) forms in stage II Fh1 β KO and CTL islets (at least 150 islets per genotype). The calculated peptide mass based on the detected m/z (m , mass; z , charge) value of the doubly charged precursor peptide ion ($[M+2H]^{2+}$) and the calculated ($[M]$ calc.) and theoretical peptide mass ($[M]$ theor.) are stated for both peptide species. Detected N- and C-terminal fragment ions are indicated in the peptide sequence, assigned in the spectrum and depicted as follows: b: N-terminal fragment ion; y: C-terminal fragment ion; *: fragment ion minus NH_3 ; and $^{2+}$: doubly charged fragment ion. Both theoretical mass (in brackets) and detected mass are given for each assigned fragment ion. Peptide fragments that include the succinated cysteine residue are highlighted in red, while unsuccinated fragments are depicted in green. Insets in (G) and (H) show the extracted ion chromatograms of the precursor peptide from a representative triplicate run of analyzed pancreatic islets. Quantification of the peptide, corresponding to residues 144–160 of Gapdh, succinated at C150, indicate that the succinated peptide was enriched by 270%, while the unmodified peptide was decreased by 70%. PE indicates the modification of cysteine residues at C150 and C154 to pyridylethyl-cysteine in the inset in (G) and only C154 in (H). Error bars represent \pm SEM. See also Figure S6 and Table S2.

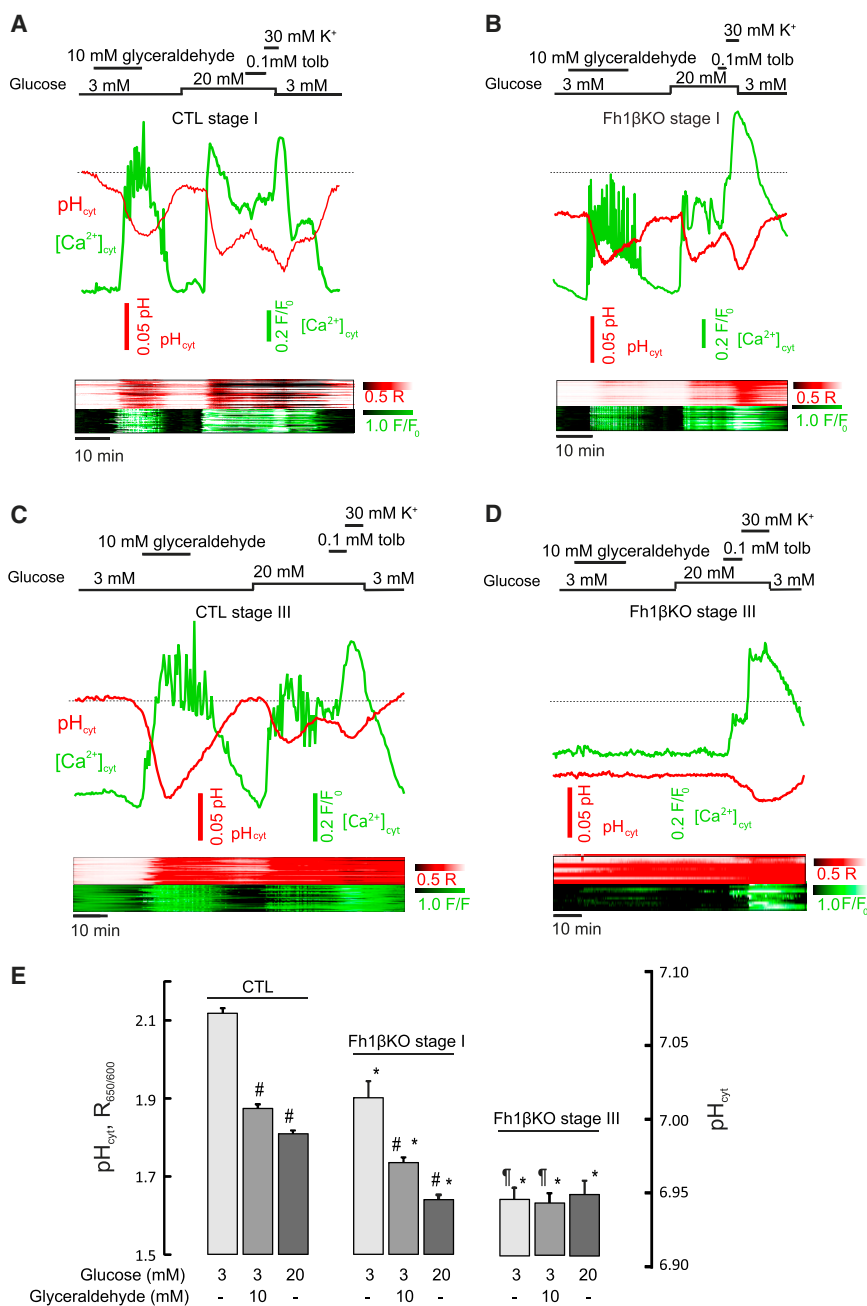


Figure 6. Effect of *Fh1* Deletion on $[Ca^{2+}]_i$ and pH_i in β Cells

(A and B) Top: simultaneous measurements of $[Ca^{2+}]_i$ (green) and pH_i (red) in β -cells within intact pancreatic islets from stage I CTL (A) and Fh1 β KO (B) mice exposed to 3 mM or 20 mM glucose, 10 mM D-glyceraldehyde, 30 mM K^+ , or 0.1 mM tolbutamide. The dashed line indicates basal pH_i . Bottom: heatmaps showing pH_i (red) and $[Ca^{2+}]_i$ (green) responses for individual cells (>20 cells within a single islet). Color intensity indicates concentration range from low (black) to high (white). See calibration bars at right.

(C and D) Same as in (A) and (B) but using islets from stage III CTL (C) and Fh1 β KO (D) mice.

(E) Mean \pm SEM fluorescence ratio (left) at 3 mM glucose, with or without 10 mM D-glyceraldehyde or 20 mM glucose in β cells from stage I (gray bars) Fh1 β KO (n = 95 cells from 2 mice) and stage III (white bars) Fh1 β KO (n = 39 cells from 4 mice) mouse islets. *p < 0.05 versus basal (3 mM) glucose and CTL (n = 163 cells from 6 mice; there was no difference between stage I and stage III CTL β cells, and data have been pooled for display). *p < 0.05 versus stage I Fh1 β KO β cells. Approximate changes in calibrated pH_i are shown (right).

of diabetes caused by a gain-of-function mutation in the K_{ATP} channel ($\beta V59M$ mice; Brereton et al., 2016). These scenarios are not mutually exclusive, and it is possible that they operate in parallel and that both contribute to the loss of ATP production and GSIS. Both these hypotheses predict that inhibition of glucose metabolism should exert a protective effect on β cell metabolism. This proved to be the case, as GSIS was restored in Fh1 β KO islets following “normoglycemic” culture or culture in high glucose with the glucokinase inhibitor mannoheptulose.

Fumarate Accumulation Leads to Protein Succination

Our data demonstrate that elevation of intracellular fumarate is associated with

from stage I Fh1 β KO mice had a higher pH_i than β cells from stage III mice. At higher plasma glucose levels, mitochondrial glucose metabolism will accelerate due to the increased substrate, leading to greater fumarate generation, acidification of both cytoplasmic and mitochondrial matrices, and a progressive impairment of ATP production. Also, acidification may influence the activity of other enzymes involved in β cell metabolism with optimal activity at alkaline pH (e.g., Bernstein and Everse, 1978; Lai and Cooper, 1986; Willson and Tipton, 1980). These effects may be aggravated by downregulation of key genes involved in mitochondrial metabolism, as seen in a mouse model

“hyperglycemia” in both mouse and human islets. We detected succination of critical cysteines in GAPDH, GMPPR, and PARK7/DJ-1 proteins in Fh1 β KO islets. Succination of GAPDH has been reported previously in adipocytes in diabetic *db/db* and *ob/ob* mice. It is a marker of impaired mitochondrial metabolism and has functional effects (Blatnik et al., 2008; Frizzell et al., 2011). Reduced PARK7/DJ1 activity is compatible with the small mitochondria (suggestive of mitochondrial fragmentation and impaired function) seen in Fh1 β KO islets. Interestingly, we found that fumarate levels are elevated in islets from T2D donors and may, via succination, explain why expression of PARK7 is

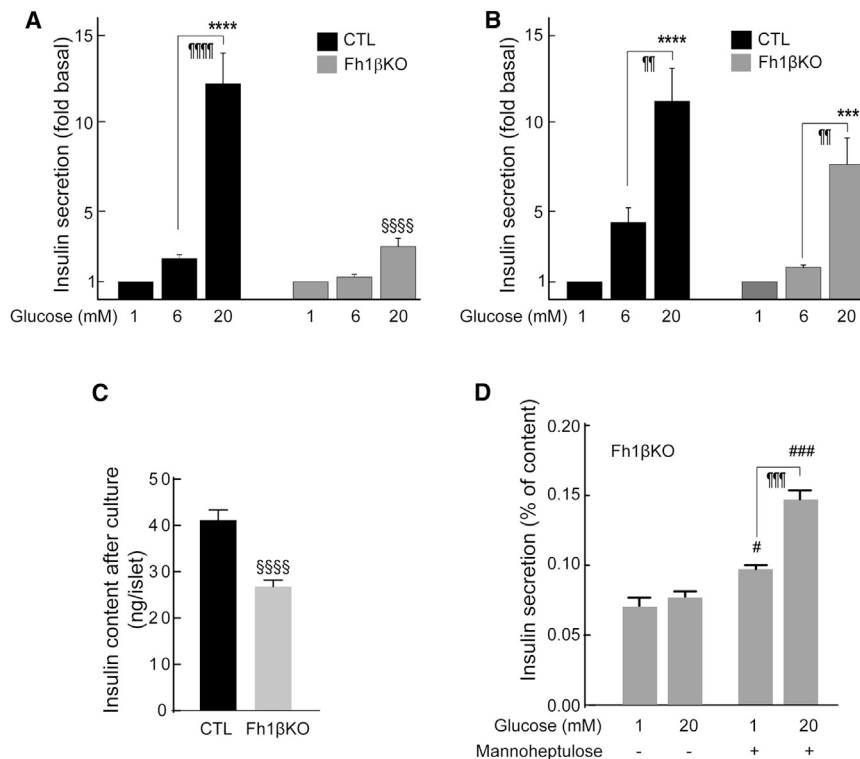


Figure 7. GSIS Dysfunction in Diabetic Fh1βKO Islets Can Be Reversed by "Normoglycemia"

(A) Insulin secretion from freshly isolated islets from stage III Fh1βKO (gray) and CTL (black) littermates at 1 mM, 6 mM, or 20 mM glucose during static incubation (n = 12–18 experimental groups of islets from at least 8 mice of each genotype in a total of 3 experiments). ****p < 0.0001 versus 1 mM glucose; ****p < 0.0001 versus 6 mM glucose; ****p < 0.0001 versus 20 mM glucose in CTL.

(B) Insulin secretion from the same islets as in (A) after 65-hr culture at 12 mM glucose. ***p < 0.001 versus 1 mM glucose; **p < 0.01 versus 6 mM glucose; ****p < 0.0001 versus 20 mM glucose in CTL.

(C) Insulin content of islets used in (B). ****p < 0.0001.

(D) Insulin secretion in islets isolated from stage III Fh1βKO mice measured in static incubations at 1 mM or 20 mM glucose after 72-hr culture at 20 mM glucose with or without 10 mM mannoheptulose (n = 3 mice). ****p < 0.001 versus 1 mM glucose; #p < 0.05, and ###p < 0.001 versus corresponding condition without prior culture in the presence of mannoheptulose.

Error bars represent ± SEM. See also Figure S7.

reduced in T2D islets (Jain et al., 2012). Importantly, it also suggests that mitochondrial metabolism is impaired in islets, leading to elevated fumarate and succination.

Conclusions

Our studies suggest a cycle in which progressive hyperglycemia in Fh1βKO mice leads to the deterioration of metabolism, culminating in the loss of GSIS and frank diabetes. Although β cells from young Fh1βKO mice are glucose responsive, subtle differences exist between Fh1βKO and CTL islets. Thus, GSIS is impaired at 6 mM glucose in Fh1βKO mice, compared to CTL mice. This defect may underlie the slight elevation in plasma glucose that precedes the more rapid deterioration in glucose tolerance. Our hypothesis is consistent with the proposal that hyperglycemia, via β cell decompensation, initiates a cycle of progressive hyperglycemia and impaired GSIS (Weir and Bonner-Weir, 2004).

In severely diabetic mice expressing the gain-of-function K_{ATP} channel mutation V59M (Brereton et al., 2014), the adverse effects on β cell function and insulin content were reversed following restoration of normoglycemia. Similarly, the severe impairment of GSIS seen in diabetic (stage III) Fh1βKO islets was almost fully corrected simply by culturing the islets under "normoglycemic" conditions. This agrees with the report that GSIS is restored in long-term T2D patients following normalization of plasma glucose levels and suppression of hepatic glucose production induced by a low-calorie diet (Lim et al., 2011).

We propose that the Fh1βKO mouse provides a valuable new model for T2D. In particular, it is not obese, and the glucose intol-

erance develops in an age-dependent fashion. Thus, it provides a useful tool for studying the progression observed in T2D and to interrogate the systemic and cellular consequences of metabolic dysfunction in the pancreatic β cell without the complications of altered diet and/or obesity.

EXPERIMENTAL PROCEDURES

See also Supplemental Experimental Procedures.

Mice

Animal experiments were conducted in accordance with the UK Animals Scientific Procedures Act (1986) and University of Oxford local ethical guidelines. We used male and female adult mice of the following strains: *Fh1^{tm1Pjpl/f}Rip2-Cre^{+/-}* (*Fh1^{tm1Pjpl/f}* crossed with *Tg(Ins2-Cre)^{23Her}* Cre recombinase, *Rip2-Cre^{+/-}*), *Fh1^{tm1Pjpl/f}Hif1α^{fl/fl}Rip2-Cre^{+/-}*, *Fh1^{tm1Pjpl/f}Rip2-Cre^{+/-}Gt(Rosa)26Sor^{tm1(CAG-FH)Pjpl}*; *Tg(Cdh16-cre)91lgr*, *Fh1^{tm1Pjpl}Gt(Rosa)26Sor^{tm1(CAG-FH)Pjpl}*; *Tg(Cdh16-cre)91lgr*, and *Fh1^{tm1Pjpl/f}Rip2-Cre^{+/-}Nrf2^{-/-}* mice (designated Fh1βKO, Fh1Hif1αβKO, Fh1βKO+FH, Fh1βKO+FH^{cyt}, and Fh1β/Nrf2KO, respectively) and littermate controls (designated CTL). The constitutive Nrf2 KO mouse was generated from an embryonic stem cell (ESC) clone obtained from Riken, Japan. Genotyping was performed by Transnetix, but primer details and PCR conditions can be obtained from J.A. NMRI and C57BL/6J mice (designated wild-type) purchased commercially were used in a few cases.

IHC

Mouse tissues were fixed in 10% neutral-buffered formalin, dehydrated, and processed for paraffin wax embedding and sectioning (3 μm). H&E sections were generated for all samples. IHC was carried out using the EnVision Kit (Dako) as per the manufacturer's protocol, with the following antibodies: FH (Autogen Bioclear), HIF1α (Cayman), insulin (MP Biomedicals), glucagon (MP Biomedicals and Sigma), and 2SC (Nagai et al., 2007).

Intraperitoneal Glucose Tolerance Test

Blood glucose levels were determined with an Accucheek Aviva meter after 16 hr of fasting and at time points following intraperitoneal injection of 2 g glucose per kilogram of body weight. Animals were culled at the end of the test, and pancreata were processed as described earlier.

Hormone Secretion and Content Measurements

Insulin secretion was measured by *in situ* pancreas perfusion or in static incubations of isolated islets (Zhang et al., 2013). Insulin content was determined in parallel from isolated islets or from mouse pancreata harvested separately.

Quantitative Imaging of ATP, Ca²⁺, and pH_i

Time-lapse imaging of the ATP/ADP ratio in mouse islets was performed using 10×–14× magnification on a Zeiss AxioZoom.V16 microscope. Islets were infected with an adenovirus (3 × 10⁴ plaque-forming units [PFUs] per islet) delivering Perceval, a recombinant sensor of ATP/ADP (Berg et al., 2009). Groups of islets isolated from CTL and Fh1βKO animals were imaged simultaneously 24 hr post-infection at glucose concentrations as indicated, with single-cell resolution. Time-lapse images were collected every 30 s, and the bath solution was perfused at 60 μL/min at 34°C.

Simultaneous time-lapse imaging of [Ca²⁺]_i and pH_i in mouse islets was performed on the inverted Zeiss AxioVert 200 microscope, equipped with the Zeiss LSM 510-META laser confocal scanning system, using a 40×/1.3 NA objective. Mouse islets were loaded with 6 μM of the Ca²⁺-sensitive dye Fluo-4 for 90 min before being transferred to a separate solution containing 6 μM of the pH-sensitive dye SNARF-5F (both dyes from Molecular Probes) for a further 50 min at room temperature and imaged using an open chamber at 34°C. The bath solution containing various stimuli was perfused continuously at the rate of 200 μL/min. The ratiometric dye SNARF-5F was excited at 543 nm, and emission was collected at 650 nm and 600 nm. Fluo-4 was excited at 488 nm and imaged at 530 nm. Images were acquired at the frequency of 0.03 Hz. The pH calibration of each trace was performed using the high K⁺-nigericin technique. Valinomycin (5 μM) was added to the extracellular solution to abolish the K⁺ gradient (Tarasov et al., 2012, 2013).

Statistical Analysis

Image sequences were analyzed (registration, background subtraction, ROI intensity versus time analysis) using open-source Fiji software (<http://fiji.sc/Fiji>). The numerical time series data were analyzed using the IgorPro package (Wavemetrics). Statistical significance of the differences between paired or unpaired samples was tested using Friedman or Kruskal-Wallis tests, respectively, with Nemenyi post hoc analysis, as implemented in the R package (R Development Core Team, 2016). Basal pH_i was calculated by taking the mean of the first 15 SNARF-5F ratio values from each cell. After glyceraldehyde and 20 mM glucose application, pH_i was calculated by taking a mean of the values when the SNARF-5F ratio reached a nadir. Differences with *p* < 0.05 were considered significant. Cells that were not active at 3 mM glucose and that responded to high glucose with the characteristic [Ca²⁺]_i oscillations were taken as β cells.

All data are given as mean ± SEM unless indicated that they are mean ± SD. Other than for image analysis, as indicated earlier, statistical significance was determined with significance set at <0.05, using either ANOVA with Tukey's multiple comparison or Student's *t* test (where indicated). Statistical significance was determined using GraphPad Prism v.6.0d (GraphPad Software, La Jolla, CA, USA; <http://www.graphpad.com>).

Human Islets and Ethics

Pancreatic islets were isolated from deceased donors under ethical approval obtained from the local human research ethics committees in both Oxford and Lund. All donors gave informed research consent. Islets were obtained from the Diabetes Research & Wellness Foundation Human Islet Isolation Facility, OCDEM, University of Oxford, and the Nordic Center for Clinical Islet Transplantation (<http://www.nordicislets.com>; Uppsala, Sweden) via the Human Tissue Laboratory at Lund University Diabetes Centre. Islets were hand picked, and their quality was assessed prior to research use. Experiments in Oxford were performed using islets from donors (5 females and 1 male) with the following parameters: age, 47.8 years ± 6.7 years; BMI,

28.4 ± 5.8. In Lund, the characteristics of ND (*n* = 31, CTL) and diabetic (*n* = 7, T2D) islet donors were as follows: age (years), 61.24 ± 10.45 (CTL) and 60.43 ± 7.72 (T2D), *p* = 0.82; sex, expressed as male/female, 14/7 (CTL) and 2/5 (T2D), *p* = 0.44; BMI, 27.11 ± 3.06 (CTL) and 28.9 ± 5.35 (T2D), *p* = 0.42; and HbA1c, 5.85 ± 0.45 (CTL) and 6.41 ± 0.56, *p* = 0.04. Data are given as mean ± SD; groups were compared using the Student's *t* test or using two-tailed Fisher's exact test.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and two tables and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2017.08.093>.

AUTHOR CONTRIBUTIONS

J.A., P.J.P., and P.R. designed the study. J.A., R.R., M.V.C., N.T., A.H., A.I.T., Q.Z., E.R., N.J.G.R., R.M.d.R., G.O., H.W.D., P.S., K.S., K.K., K.I., B.M.K., J.T.-R., H.M., A.C., T.S., and P.J.P. collected and analyzed the data. A.L., A.S., C.W.P., N.F., and P.R. analyzed data and contributed expertise. P.J.P., M.V.C., A.S., A.I.T., and J.A. made the final figures. J.A., A.S., P.J.P., P.R., and F.M.A. wrote the manuscript, which was critically edited by all authors.

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This paper is a tribute to Dr. Patrick John Pollard, who died unexpectedly in June 2015. He was an enthusiastic and exceptionally gifted scientist. We remember him fondly as a friend and colleague who made important contributions, not only to this study, but also to the field of cancer metabolism.

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